



TITLE:

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Evolutionary Relationships and Reproductive Isolating Mechanisms in the Rice Frog (*Fejervarya limnocharis*) Species Complex from Sri Lanka, Thailand, Taiwan and Japan, Inferred from mtDNA Gene Sequences, Allozymes, and Crossing Experiments

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The rice frog (*Fejervarya limnocharis*) species complex is widely distributed, from India to Japan, and most prevalently in Southeast Asia. Conspicuous morphological variation has been reported for this species complex throughout its distribution range. In the present study, we used mtDNA gene sequence and allozyme analyses to infer evolutionary affinities within this species complex using eight populations (Sri Lanka; Bangkok and Ranong in Thailand; Taiwan; and Hiroshima, Okinawa, Ishigaki and Iriomote in Japan). We also conducted crossing experiments among four populations from Japan, Thailand, and Sri Lanka in order to find out more about the reproductive isolating mechanisms that might exist among the East, Southeast, and South Asian populations of this species complex. The crossing experiments revealed that the Sri Lanka population is reproductively isolated from the Hiroshima, Bangkok, and Ranong populations by complete hybrid inviability, and that the Bangkok population may be reproductively isolated from the Hiroshima population by partial hybrid inviability. Thus, it is not unreasonable to regard the Sri Lanka population as a species separated from *F. limnocharis*. The mtDNA and allozyme data showed that the Ranong population is most closely related to the Bangkok population in nuclear genome, but more similar to the Okinawa and Taiwan populations in mtDNA genome. The present, preliminary survey may raise questions about the species status of these particular populations and also about the nature of the biological species concept.

Key words: speciation, reproductive isolation, genetic divergence, molecular phylogeny, *Fejervarya*, Asia

INTRODUCTION

According to Mayr (1969), the simple definition of species is “groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups.” Species are also viewed as “the largest and most inclusive ... reproductive community of sexual and cross-fertilizing individuals which share in a common gene

pool.” Based on the biological species concept, if two taxa are reproductively isolated from each other and cannot interbreed, they are considered different species. It may be that different species are first isolated from each other, either completely or incompletely, by gametic isolation: when the gametic isolation is incomplete, the two species are completely or incompletely isolated by hybrid inviability; when the hybrid inviability is incomplete, the two species are completely isolated by hybrid sterility (Sumida *et al.*, 2003). The biological species concept has been challenged by proponents of the phylogenetic and evolutionary species concepts (Hanken, 1999). These concepts generally place less emphasis on reproductive isolation and much greater

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emphasis on phylogenetic history and assessment of the likely evolutionary independence of particular lineage (Frost and Hillis, 1990). These two species concepts are not always consonant when applied to natural systems.

The rice frog (*Fejervarya limnocharis*) is one of the most widely distributed species of Asian frogs. It can be found in every country of East, Southeast, and South Asia in a range that extends across western Japan, Taiwan, China, the Malay Peninsula, Bangladesh, Nepal, the Philippines, Indonesia, Sri Lanka, and India to Pakistan. There are numerous papers dealing with the systematics of the *F. limnocharis* species complex. *Fejervarya limnocharis* was first described in Java (Gravenhorst, 1829; Wiegmann, 1834). *Fejervarya limnocharis* from the Philippines and Sri Lanka were recognized as subspecies and named *F. limnocharis vittigera* and *F. limnocharis greenii*, respectively (Inger, 1954; Kirtisinghe, 1957), and later elevated to valid species as *F. vittigera* and *F. greenii*, respectively (Dubois, 1984). Dubois (1975) insisted that Nepalese "*F. limnocharis*" could be separated into four species, including *F. nepalensis*, *F. pierrei*, and *F. syhadrensis*, and he also (1984) suggested *F. limnocharis nicobariensis* as a synonym for *F. andamanensis*. Dubois (1984, 1987, 1992) enumerated even more species in southern India, and surmised that the whole group consisted of at least 15 species, and probably many more. Dutta (1997) reported that the *F. limnocharis* complex included nine nominal species in India. Some (*F. pierrei* and *F. nepalensis*) have been separated on the basis of their call pattern. Toda *et al.* (1997) demonstrated with allozyme analysis the presence of two taxa occurring in sympatry in Java. Veith *et al.* (2001) described one of them as a new taxon known only in Java, and named it *F. iskandari*. Maeda and Matsui (1999) regarded the Okinawan Sakishima-Island

populations of *F. limnocharis*, including Ishigaki and Iriomote Islands, as a distinct species on the basis of morphological, acoustic and genetic differences. Several investigators have reported conspicuous morphological and genetic divergence of the Sakishima-Island populations from other populations of this species (Kuramoto, 1979; Nishioka and Sumida, 1990; Sumida *et al.*, 2002; Toda *et al.*, 1997; Toda, 1999). On the basis of morphological observations, allozyme data, molecular data and crossing experiments, Sumida *et al.* (2002) suggested that it is reasonable to regard the Sakishima-Island populations as a subspecies of *F. limnocharis*. Dubois and Ohler (2000) suggested that the group of frogs previously known as the "*Rana limnocharis* group" (Annandale, 1917) be regarded as a distinct genus, *Fejervarya*. So far, a total of 31 species have been listed in the genus *Fejervarya* (Frost, 2004), but as has been pointed by Dubois (1984), the revision of this group is difficult. Thus, further systematic work and comprehensive surveys are needed to elucidate all aspects of speciation within this complex.

In the present study, mtDNA gene sequence and allozyme analyses were carried out using eight populations from Sri Lanka, Thailand, Taiwan, and Japan, in order to shed light on genetic differences within the *F. limnocharis* complex. In addition, crossing experiments were carried out among four populations of *F. limnocharis* from Sri Lanka, Thailand, and Japan in order to elucidate reproductive isolating mechanisms among populations of this species complex.

MATERIALS AND METHODS

Specimens

A total of 63 frogs belonging to eight populations of *F.*

Table 1. Specimens of *Fejervarya limnocharis* and *F. cancrivora* used in the present study and haplotypes of nucleotide sequences of the 12S and 16S rRNA genes

Species	Collecting station		No. of frogs (Body length ^a)			Haplotypes (Accession No.)	
	Country	Locality	Total	Female	Male	12S rRNA gene	16S rRNA gene
<i>Fejervarya limnocharis</i>	Japan	Hiroshima*	11	7 (33.0–46.0, 41.6)	4 (31.0–39.0, 36.3)	Hiro (AB070725 ^b)	Hiro (AB070732 ^b)
	Japan	Okinawa	18	8 (34.5–40.8, 37.7)	10 (32.0–38.5, 35.8)	Okin (AB070726 ^b)	Okin(1) (AB070733 ^b) Okin(2) (AB070734 ^b)
	Japan	Ishigaki	3	2 (56.7–58.0, 57.4)	1 (52.0)	Ishi (AB070727 ^b)	Ishi (AB070735 ^b)
	Japan	Iriomote	5	0	5 (46.0–55.0, 50.4)	Irio (AB070728 ^b)	Irio (AB070736 ^b)
	Taiwan	Chiai	8	2 (38.4–42.0, 40.0)	6 (30.0–36.0, 33.5)	Taiw (AB070729 ^b)	Taiw (AB070737 ^b)
	Thailand	Bangkok*	5	3 (48.0–54.4, 50.4)	2 (42.0–42.2, 42.1)	Bang (AB162441)	Bang (AB162444)
	Thailand	Ranong*	2	1 (50.0)	1 (42.8)	Rano (AB162442)	Rano (AB162445)
	Sri Lanka	Bentota*	3	1 (31.5)	2 (27.0–29.8, 28.4)	Sri (AB162443)	Sri (AB162446)
	Philippines	Manila	8	5 (49.0–68.0, 55.4)	3 (45.0–55.0, 50.3)	Cancri (AB070730 ^b)	Cancri (AB070738 ^b)
Total			63	29	34		

Asterisks show populations used for crossing experiments. ^a Range and average (mm). ^b Data from Sumida *et al.* (2002).

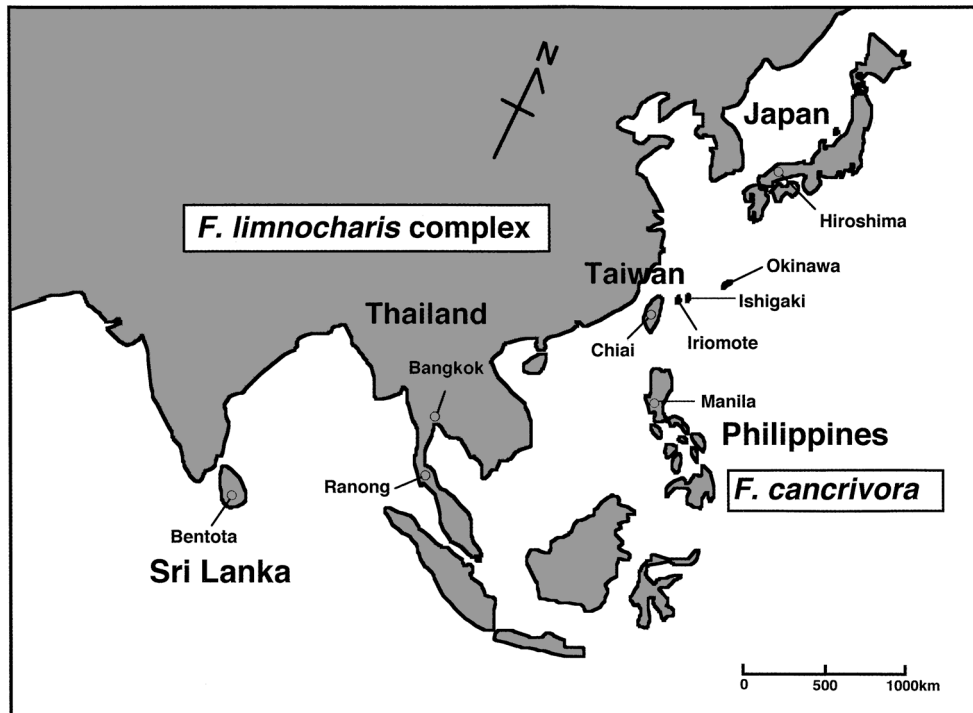


Fig. 1. Map showing the locations of eight populations of *F. limnocharis* and one population of *F. cancrivora* used in this study.

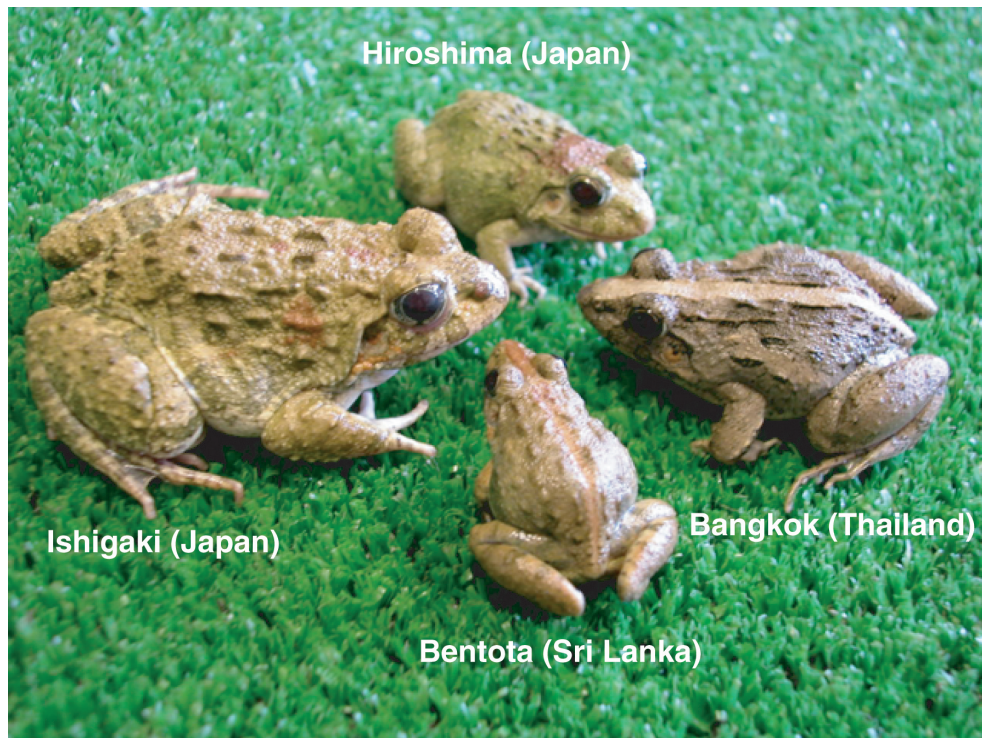


Fig. 2. Individuals from four populations (two from Japan; Thailand; Sri Lanka) of the *Fejervarya limnocharis* complex. Magnification 1.0X. This complex shows polymorphism for the mid-dorsal stripe, which is inherited as a dominant character but cannot be used as a diagnostic marker.

limnocharis from Japan, Taiwan, Thailand and Sri Lanka and one population of *F. cancrivora* from the Philippines were used in the present study (Table 1; Figs. 1, 2). Thirteen sequences of the 16S rRNA gene were obtained for 12 *Fejervarya* species from available

database, and used for phylogenetic analysis (see Fig. 10).

mtDNA sources and sequencing

Total genomic DNA for PCR was extracted from clipped toes

using a DNA extraction kit (DNeasy Tissue Kit, QIAGEN) according to the manufacturer's protocol. Primers FS01 and R16 designed by Sumida *et al.* (1998) were used for the amplification and sequencing of a 422-bp segment of the mitochondrial 12S rRNA gene corresponding to sites 2816–3225 in the *R. catesbeiana* sequence (Yoneyama, 1987). PCR primers F51 and R51 designed by Sumida *et al.* (2002) were used to amplify a 569-bp segment of the mitochondrial 16S rRNA gene corresponding to sites 3976–4549 in the *X. laevis* sequence reported by Roe *et al.* (1985). PCR mixtures were prepared using the TaKaRa Taq™ Kit as recommended by the manufacturer in a final volume of 50 µl. The 12S and 16S rRNA genes were amplified by 30 cycles, each cycle consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. PCR products were purified by MicroSpin™ S-300 HR Columns (Pharmacia Biotech) and used for sequencing. Purified DNA was sequenced by the DyeDeoxy™ Terminator Cycle Sequencing method using a 373A DNA Sequencing System Ver. 1.2 (ABI). Nucleotide sequences were analyzed using DNAsIS (Ver. 3.2, Hitachi Software Engineering) and CLUSTALW (Thompson *et al.*, 1994) for alignment. Nucleotide sequences were deposited in the DDBJ database under accession nos. AB162441–AB162446 (Table 1).

Sequence data analysis

Alignments for DNA sequences were determined on the basis of maximum nucleotide similarity using CLUSTAL X (Thompson *et al.*, 1997). To exclude gaps and ambiguous sites, we revised the alignments with GBlocks 0.91b (Castresana, 2000) using the default settings. In this manner, we constructed two sets of aligned sequences. The first comprised the concatenated 12S and 16S rRNA sequences for phylogenetic analyses of eight populations of *F. limnocharis*, with *Fejervarya cancrivora* from the Philippines as an outgroup (Sumida *et al.*, 2002). The second was the 16S data for phylogenetic analyses of nine populations of *F. limnocharis*, one

population from each of 11 *Fejervarya* species, and two populations of *F. syhadrensis*, with *Hoplobatrachus tigerinus* as an outgroup. Sequence divergences were calculated by Kimura's two-parameter model (Kimura, 1980) with a transition to transversion ratio of 2.0, and phylogenetic relationships were estimated by maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) methods, using PAUP* 4.0b10 (Swofford, 2002). The ML and NJ analyses were conducted using substitution models and parameters estimated by Modeltest 3.7 (Posada and Crandall, 1998). The MP tree was constructed by a heuristic search with 10 replicates, using simple sequence addition and tree bisection reconnection (TBR). This tree was then used as a starting tree for the ML analysis, which was conducted by heuristic search using as-is sequence addition and TBR. The reliabilities of the resulting trees were evaluated by non-parametric bootstrap statistics (BP) with 1,000 pseudoreplicates. Bayesian inference (BI) was conducted with MrBayes Ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) using the GTR+I+G substitution model with 1,500,000 generations, sampling of trees every 100th generation, and a burn-in length of 500,000. Burn-in was determined by checking convergences of $-\log$ likelihood ($-\ln L$) values using the 'sump' command implemented in MrBayes Ver. 3.1.2.

Allozyme analysis

Fourteen enzymes and two blood proteins were analyzed using starch-gel electrophoresis carried out according to the method described by Nishioka *et al.* (1980, 1992) (Table 2). Each enzyme was detected by the agar-overlay method outlined by Harris and Hopkinson (1976). Blood proteins were detected by the amido-black staining method (Nishioka *et al.*, 1980; Sumida and Nishioka, 1994). Genetic distances were calculated following Nei (1972, 1975, 1987), and Cavalli-Sforza chord measures were estimated by the method of Cavalli-Sforza and Edwards (1967). The neighbor-joining (NJ)

Table 2. Enzymes and blood proteins, and number of phenotypes and alleles at each locus, analyzed for *Fejervarya limnocharis* and *F. cancrivora*

Enzymes or blood proteins	E.C.No.	Abbreviation	No. of locus	Locus	No. of phenotypes	No. of alleles
Aspartate aminotransferase	2.6.1.1	AAT	2	AAT-1	2	2
				AAT-2	2	2
Adenosine deaminase	3.5.4.4	ADA	1	ADA	8	8
Adenylate kinase	2.7.4.3	AK	1	AK	2	2
Creatine kinase	2.7.3.2	CK	1	CK	2	2
Fumarate hydratase	4.2.1.2	FUM	1	FUM	4	4
α -Glycerophosphate dehydrogenase	1.1.1.8	α -GDH	1	α -GDH	3	3
Glucose phosphate isomerase	5.3.1.9	GPI	1	GPI	6	5
Isocitrate dehydrogenase	1.1.1.42	IDH	2	IDH-1	4	4
				IDH-2	2	2
Lactate dehydrogenase	1.1.1.27	LDH	2	LDH-A	3	3
				LDH-B	6	6
Malate dehydrogenase	1.1.1.37	MDH	2	MDH-1	7	6
				MDH-2	3	3
Malic enzyme	1.1.1.40	ME	2	ME-1	3	3
				ME-2	4	3
Mannose phosphate isomerase	5.3.1.8	MPI	1	MPI	6	5
6-Phosphogluconate dehydrogenase	1.1.1.44	6-PGD	1	6-PGD	5	4
Peptidase	3.4.3.1	PEP	4	PEP-A	4	3
				PEP-B	4	4
				PEP-C	5	4
				PEP-D	6	4
Serum albumin	—	Alb	1	Alb	4	3
Hemoglobin	—	Hb	2	Hb-I	4	4
				Hb-II	5	5
Total (Average)			25		4.2	3.8

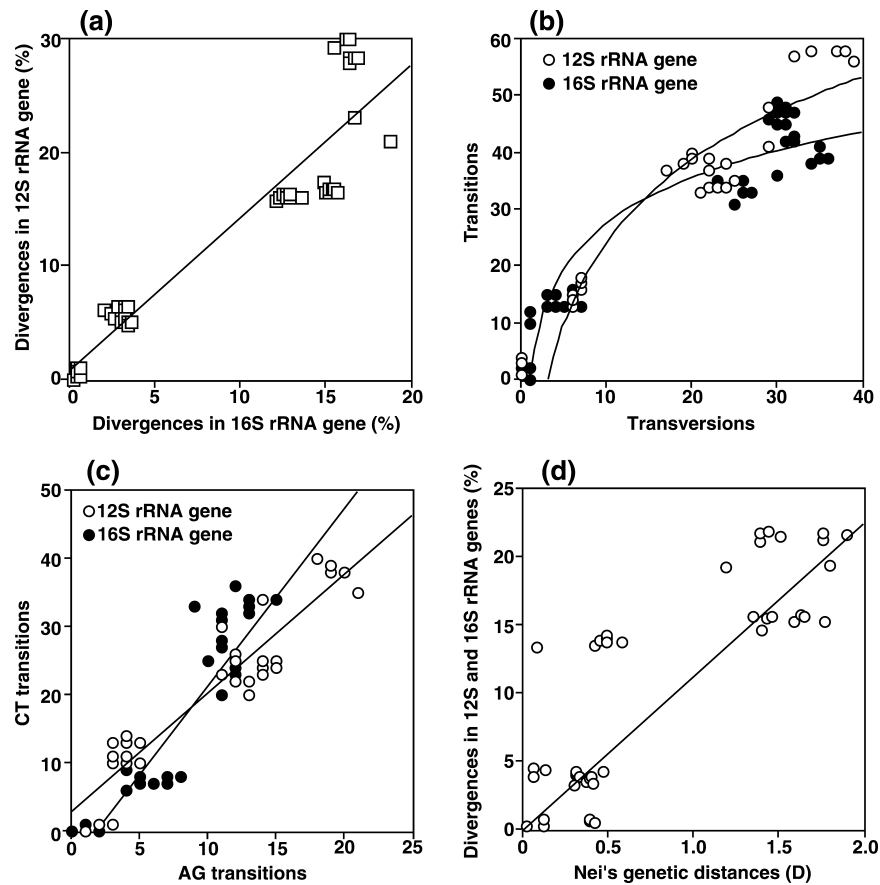


Fig. 3. (a) Relationship between percent sequence divergences of the 12S rRNA gene and the 16S rRNA gene. (b) Relationship between transitions and transversions for the 12S and 16S rRNA genes. (c) Relationship between CT and AG transitions for the 12S and 16S rRNA genes. (d) Relationship between percent sequence divergences of the 12S and 16S rRNA genes and Nei's genetic distances.

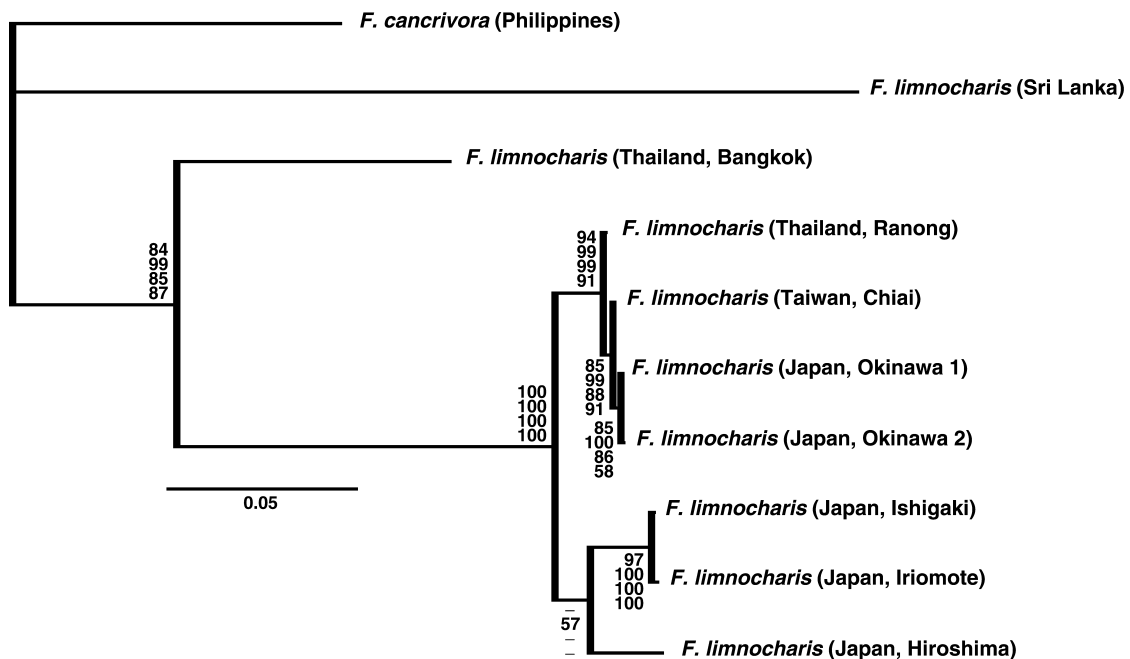


Fig. 4. Phylogenetic tree constructed by the maximum likelihood method, based on nucleotide sequences of a 422-bp segment of the 12S rRNA gene and a 569-bp segment of the 16S rRNA gene from eight populations of *F. limnocharis* and one population of *F. cancrivora* as an outgroup. Numbers near nodes are bootstrap supports (>50%) given in the order of ML, BI, MP, and NJ analyses. The scale bar represents branch length in terms of nucleotide substitutions per site.

method (Saitou and Nei, 1987) was employed to infer phylogenetic relationships among taxa on the basis of Nei's uncorrected genetic distances (1972), using a program included in PHYLIP Ver. 3.65 (Felsenstein, 2005). *Fejervarya cancrivora* from the Philippines was used as an outgroup for constructing the phylogenetic tree.

Crossing experiments and chromosome analysis

Crossing experiments were carried out among four populations of *F. limnocharis* by the routine method of artificial insemination (Table 1) (Kawamura *et al.*, 1981). Each female was induced to ovulate by injection into the body cavity of a salt solution containing mashed bullfrog pituitary. A sperm suspension was made by crushing a testis removed from each male in a small quantity of distilled water. Embryos and tadpoles were raised in Cl-free tapwater. Tadpoles were usually fed on boiled spinach, and metamorphosed frogs were usually fed on crickets. Chromosomes were observed in the tail tips of the tadpoles using the squash method after pretreatment with distilled water (Nishioka, 1972). The tail tips were cut off, immersed in distilled water for 60–90 min, stained with 1% orcein in 45% acetic acid for 30–60 min on glass slides, and squashed under cover glasses after being heated for 20–30 sec. Ploidy was determined by counting the chromosome number in the metaphase spreads.

RESULTS

mtDNA sequence data

The concatenated data used for analyses were 991 bp long, consisting of 422 bp of 12S and 569 bp of 16S. Table 5 shows the nucleotide sequence divergences and the number of nucleotide substitutions for each pair of haplotypes of the 12S and 16S sequences for *F. limnocharis* and *F. cancrivora*. The 12S alignment contained 121 variable sites, of which 63 were parsimoniously informative; the 16S alignment contained 118 variable sites, of which 58 were informative. The sequence divergence of 12S was about 1.4 times that of 16S (Fig. 3a). The frequency of transitions in 12S and 16S appeared to plateau in paired comparisons between the ingroup *F. limnocharis* and outgroup *F. cancrivora*, where multiple substitutions occurred at the same site (Fig. 3b). Comparison of the 12S and 16S haplotypes between ingroup taxa and the outgroup showed more numerous C↔T than A↔G transitions (Fig. 3c). Sequence divergences between *F. cancrivora* and the seven populations from Thailand, Taiwan, and Japan were 14.6–15.7%

Table 3. Percent divergences estimated by Kimura's two-parameter model (below diagonal) and percentages of transition sites (above diagonal) among haplotypes of 12S and 16S rRNA gene sequences in *Fejervarya limnocharis* and *F. cancrivora*

Haplotype	Hiro	Okin(1)	Okin(2)	Ishi	Irio	Taiw	Thai(B)	Thai(R)	Sri	Cancri
Hiro	–	72.1 (31/12)	73.8 (31/11)	77.1 (27/8/1*)	77.8 (28/8/1*)	73.2 (30/11)	59.1 (68/47/7*)	68.9 (31/14)	59.1 (101/70/25*)	59.3 (80/55/13*)
Okin(1)	4.6	–	0 (0/1)	73.7 (28/10/1*)	74.4 (29/10/1)	75.0 (3/1)	60.2 (71/47/7*)	85.7 (6/1/2*)	62.3 (104/63/25*)	56.5 (74/59/13*)
Okin(2)	4.5	0.1	–	75.7 (28/9/1*)	76.3 (29/9/1*)	100 (3/0)	61.3 (73/46/7*)	100 (6/0/2*)	62.7 (104/62/25*)	56.1 (74/58/13*)
Ishi	3.7	4.1	4.0	–	100 (3/0)	75.0 (27/9/1*)	62.2 (74/45/6*)	70.7 (29/12/1*)	60.9 (106/68/26*)	57.5 (77/57/14*)
Irio	3.9	4.2	4.1	0.3	–	75.7 (28/9/1*)	61.5 (72/45/6*)	71.4 (30/12/1*)	60.4 (90/59/23*)	60.0 (78/52/13*)
Taiw	4.4	0.4	0.3	3.9	4.0	–	61.0 (72/46/6*)	100 (5/0/2*)	60.4 (90/59/23*)	60.0 (78/52/13*)
Thai(B)	13.8	14.2	14.0	13.8	13.5	13.9	–	61.1 (69/44/7*)	62.4 (106/64/26*)	56.9 (70/53/16*)
Thai(R)	4.3	0.7	0.6	3.8	3.9	0.5	13.4	–	61.7 (103/64/25*)	56.3 (72/56/13*)
Sri	21.6	21.4	21.2	21.9	21.7	21.5	19.3	21.1	–	60.9 (95/61/32*)
Cancri	15.7	15.6	15.5	15.6	15.3	15.6	14.6	15.2	19.4	–

Values in parentheses are the number of transitions (Ts)/ number of transversions (Tv)/ number of gaps (G*).

Table 4. Nei's genetic distances (below diagonal) and Cavalli-Sforza chord measures (above diagonal) among nine populations of *Fejervarya limnocharis* and *F. cancrivora*

Haplotype	Hiro	Okin	Ishi	Irio	Taiw	Bang	Rano	Sri	Cancri
Hiro	–	0.079	0.465	0.479	0.149	0.628	0.517	1.199	1.130
Okin	0.059	–	0.393	0.411	0.181	0.579	0.471	1.202	1.091
Ishi	0.387	0.310	–	0.024	0.404	0.557	0.477	1.100	1.150
Irio	0.404	0.314	0.017	–	0.404	0.497	0.455	1.070	1.172
Taiw	0.103	0.121	0.328	0.328	–	0.528	0.490	1.134	1.075
Bang	0.582	0.487	0.494	0.422	0.447	–	0.148	1.009	1.091
Rano	0.466	0.390	0.407	0.373	0.419	0.083	–	1.073	1.130
Sri	1.898	1.761	1.441	1.394	1.506	1.185	1.359	–	1.202
Cancri	1.628	1.433	1.650	1.768	1.378	1.398	1.592	1.801	–

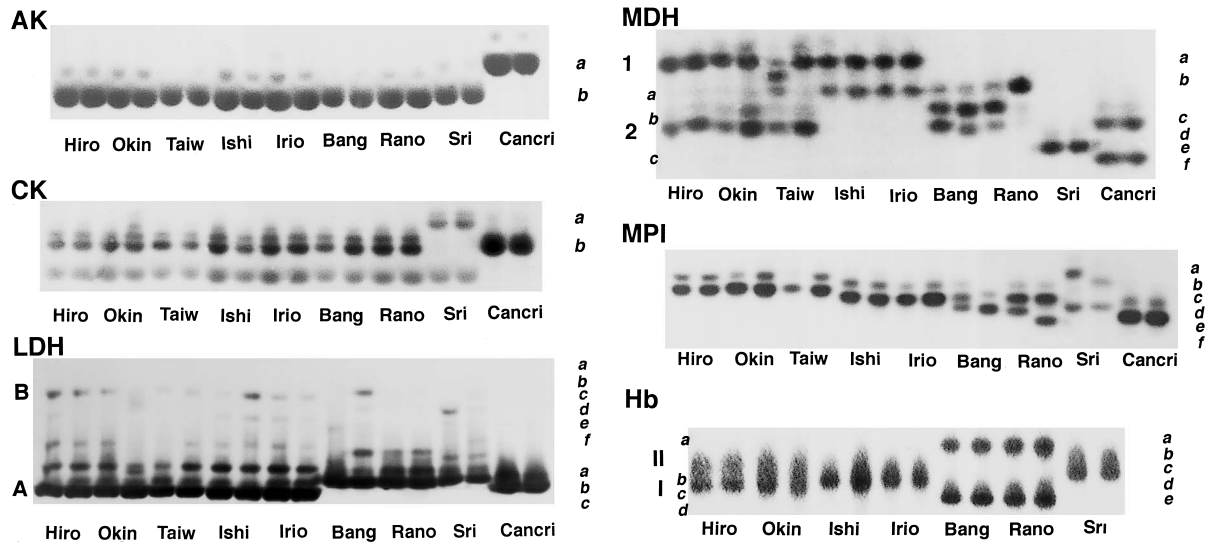


Fig. 5. Electrophoretic patterns of five enzymes and one blood protein for eight populations of *F. limnocharis* and one population of *F. cancrivora*.

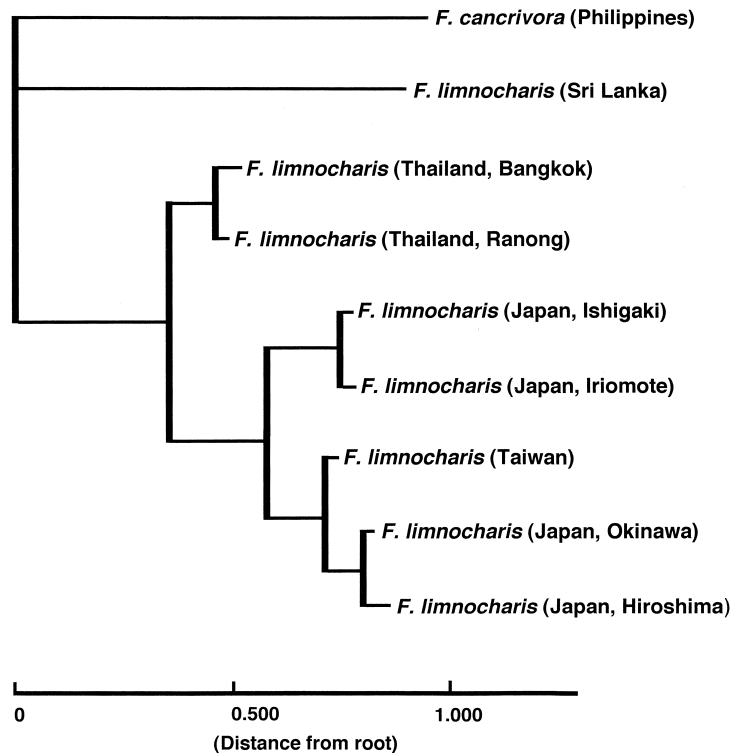


Fig. 6. Phylogenetic tree constructed by the neighbor-joining method based on Nei's genetic distances calculated from allelic frequencies at 24 loci from eight populations of *F. limnocharis* and one population of *F. cancrivora* as an outgroup.

($x=15.4\%$), with 123–135 nucleotide substitutions; those between the Sri Lanka population and the same seven populations were 19.3–21.9% ($x=21.2\%$), with 149–174 nucleotide substitutions; and that between the Sri Lanka population and *F. cancrivora* was 19.4%, with 156 nucleotide substitutions (Table 3). Sequence divergences between the Bangkok population and five populations from Taiwan and Japan were 13.5–14.2% ($x=13.9\%$), with 115–119

nucleotide substitutions (Table 3). In contrast, sequence divergences between the Ranong population and the five populations from Taiwan and Japan were only 0.5–4.3% ($x=2.3\%$), with 5–45 nucleotide substitutions, and that between the Bangkok and Ranong populations from Thailand was 13.4%, with 113 nucleotide substitutions (Table 3).

In the ML tree generated from the combined 12S and

16S data, *F. limnocharis* from the Sri Lanka population formed a basal polytomy with the outgroup, *F. cancrivora*, separate from all other ingroup taxa (Fig. 4). The remaining *F. limnocharis* all fell within a clade supported by 84–99%

bootstrap support, with the haplotype from the Bangkok population basal to all of the rest. The other seven populations of *F. limnocharis* formed three main clades (BP=100%), the first comprising the Ranong, Taiwan and Okinawa popula-

Table 5. Developmental capacity and sex of the hybrid offspring among four populations of *Fejervarya limnocharis*, and of control offspring

Parents		No. of eggs	No. of normally cleaved eggs (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of normally feeding tadpoles (%)	No. of 20-day-old tadpoles		No. of metamorphosed frogs (%)
Female	Male						Normal (%)	Underdeveloped (%)	
Hiro.1	Hiro.1	67	46(68.7)	25(37.3)	22(32.8)	22(32.8)	20(29.9)	0	20(29.9)
Hiro.2	Hiro.1	95	58(61.1)	46(48.4)	42(44.2)	41(43.2)	33(34.7)	0	32(33.7)
Hiro.3	Hiro.1	124	54(43.5)	21(16.9)	16(12.9)	16(12.9)	14(11.3)	0	13(10.5)
Hiro.4	Hiro.1	91	55(60.4)	28(30.8)	23(25.3)	23(25.3)	22(24.2)	0	22(24.2)
Hiro.5	Hiro.1	154	125(81.2)	76(49.4)	48(31.2)	42(27.3)	41(26.6)	0	37(24.0)
Hiro.6	Hiro.1	101	81(80.2)	63(62.4)	59(58.4)	52(51.5)	51(50.5)	0	51(50.5)
Hiro.7	Hiro.2	49	47(95.9)	46(93.9)	44(89.8)	33(67.3)	33(67.3)	0	27(55.1)
Total		681	466(68.4)	305(44.8)	254(37.3)	229(33.6)	214(31.4)	0	202(29.7)
Hiro.1	Bang.1	213	124(58.2)	78(36.6)	62(29.1)	59(27.7)	6(2.8)	12(5.6)	12(5.6)
Hiro.2	Bang.1	215	112(52.1)	76(35.3)	67(31.2)	61(28.4)	2(0.9)	39(18.1)	22(10.2)
Hiro.3	Bang.1	164	143(87.2)	82(50.0)	59(36.0)	52(31.7)	7(4.3)	28(17.1)	23(14.0)
Hiro.4	Bang.1	28	8(28.6)	5(17.9)	3(10.7)	3(10.7)	2(7.1)	0	2(7.1)
Hiro.5	Bang.1	78	42(53.8)	25(32.1)	18(23.1)	14(17.9)	3(3.8)	10(12.8)	6(7.7)
Hiro.6	Bang.1	200	176(88.0)	159(79.5)	144(72.0)	129(64.5)	5(2.5)	33(16.5)	21(10.5)
Hiro.7	Bang.2	110	110(100)	99(90.0)	96(87.3)	70(63.6)	0	60(54.5)	31(28.2)
Total		1008	715(70.9)	524(52.0)	449(44.5)	388(38.5)	25(2.5)	182(18.1)	117(11.6)
Hiro.4	Rano.1	27	13(48.1)	4(14.8)	4(14.8)	2(7.4)	2(7.4)	0	2(7.4)
Hiro.5	Rano.1	290	223(76.9)	118(40.7)	101(34.8)	79(27.2)	66(22.8)	0	63(21.7)
Total		317	236(74.4)	122(38.5)	105(33.1)	81(25.6)	68(21.5)	0	65(20.5)
Hiro.1	Sri 1	187	71(38.0)	25(13.4)	13(7.0)	1(0.5)	0	0	–
Hiro.2	Sri 1	110	15(13.6)	1(0.9)	1(0.9)	0	–	–	–
Hiro.3	Sri 1	223	81(36.3)	16(7.2)	12(5.4)	0	–	–	–
Hiro.4	Sri 1	121	10(8.3)	0	–	–	–	–	–
Hiro.5	Sri 1	223	56(25.1)	3(1.3)	2(0.9)	0	–	–	–
Hiro.6	Sri 1	213	65(30.5)	29(13.6)	16(7.5)	5(2.3)	0	0	–
Total		1077	298(27.7)	74(6.9)	44(4.1)	6(0.6)	0	0	–
Bang.1	Bang.1	112	97(86.6)	68(60.7)	67(59.8)	64(57.1)	62(55.4)	0	57(50.9)
Bang.2	Bang.1	269	219(81.4)	160(59.5)	149(55.4)	148(55.0)	130(48.3)	0	127(47.2)
Total		381	316(82.9)	228(59.8)	216(56.7)	212(55.6)	192(50.4)	0	184(48.3)
Bang.1	Hiro.1	16	12(75.0)	10(62.5)	10(62.5)	10(62.5)	0	9(56.3)	8(50.0)
Bang.2	Hiro.1	3	1(33.3)	1(33.3)	1(33.3)	1(33.3)	0	1(33.3)	1(33.3)
Bang.2	Hiro.2	45	19(42.2)	15(33.3)	11(24.4)	11(24.4)	0	10(22.2)	9(20.0)
Total		64	32(50.0)	26(40.6)	22(34.4)	22(34.4)	0	20(31.3)	18(28.1)
Bang.2	Sri 1	284	25(8.8)	15(5.3)	9(3.2)	0	–	–	–
Rano.1	Rano.1	259	185(71.4)	148(57.1)	140(54.1)	140(54.1)	136(52.5)	0	133(51.4)
Rano.2	Rano.2	146	144(98.6)	126(86.3)	113(77.4)	88(60.3)	85(58.2)	0	43(29.5)
Rano.3	Rano.2	178	166(93.3)	162(91.0)	160(89.9)	154(86.5)	151(84.8)	0	117(65.7)
Total		583	495(84.9)	436(74.8)	413(70.8)	382(65.5)	372(63.8)	0	293(50.3)
Rano.1	Hiro.1	41	26(63.4)	24(58.5)	24(58.5)	23(56.1)	23(56.1)	0	23(56.1)
Rano.1	Hiro.2	115	90(78.3)	71(61.7)	65(56.5)	65(56.5)	58(50.4)	0	58(50.4)
Rano.2	Hiro.3	114	107(93.9)	83(72.8)	70(61.4)	58(50.9)	56(49.1)	0	48(42.1)
Rano.3	Hiro.3	144	140(97.2)	128(88.9)	125(86.8)	123(85.4)	121(84.0)	0	113(78.5)
Total		414	363(87.7)	306(73.9)	284(68.6)	269(65.0)	258(62.3)	0	242(58.5)
Rano.1	Bang.1	282	218(77.3)	207(73.4)	171(60.6)	170(60.3)	0	71(25.2)	49(17.4)
Rano.2	Bang.3	161	159(98.8)	131(81.4)	121(75.2)	101(62.7)	0	96(59.6)	18(11.2)
Rano.3	Bang.3	165	164(99.4)	162(98.2)	161(97.6)	159(96.4)	0	157(95.2)	9(5.5)
Total		608	541(89.0)	500(82.2)	453(74.5)	430(70.7)	0	324(53.3)	76(12.5)
Rano.1	Sri 1	307	64(20.8)	44(14.3)	28(9.1)	0	–	–	–

tions (BP=91–99%), the second comprising the Ishigaki and Iriomote populations (BP=97–100%), and the third comprising the Hiroshima population (Fig. 4).

Allozyme data

The electrophoretic patterns of 14 enzymes and two blood proteins showed species-specific or population-specific bands (Fig. 5). Nei's (1972) genetic distances and Cavalli-Sforza chord measures were estimated from the gene frequencies at 24 enzyme and blood protein loci (Table 4). Nei's genetic distances between *F. cancrivora* and the seven populations from Thailand, Taiwan, and Japan ranged from 1.378 to 1.768, with an average value of 1.550, whereas those between the Sri Lanka population and the same seven populations ranged from 1.185 to 1.898, with an average value of 1.506, and that between the Sri Lanka population and *F. cancrivora* was 1.801 (Table 4).

Genetic distances between two populations from Thailand and five from Taiwan and Japan ranged from 0.373 to 0.582, with an average value of 0.449, and that between the Bangkok and Ranong populations from Thailand was only 0.083 (Table 4). In the NJ tree based on genetic distances, the outgroup, *F. cancrivora*, formed a polytomy with *F. limnocharis* from the Sri Lanka population and a clade comprising the rest of the ingroup (*F. limnocharis*), within which the Bangkok and Ranong populations first diverged, followed by the Ishigaki and Iriomote populations, the Taiwan population, and finally the Okinawa and Hiroshima populations (Fig. 6).

When the 12S and 16S divergences were plotted against Nei's genetic distance for all pairwise comparisons of nine populations, the rates of sequence divergence were approximately 10 times those of the allozyme-based genetic distances for almost all pairwise comparisons, except for

Table 6. Ploidy and sex of the hybrid offspring among three populations of *Fejervarya limnocharis*, and of control offspring

Parents		No. of	No. of analyzed tadpoles (%)						No. of metamorphosed frogs (%)						Sex of frogs examined (%)									
		20-day-old																						
		tadpoles	Total	Normal		Under-developed		Total	Normal			Under-developed		Total	2n			3n						
Female	Male			2n	3n	2n	3n		2n	3n	Unknown	2n	3n		♀	♂	Unknown	♀	♂					
Hiro.1	Hiro.1	20	20	20	0	—	—	20	20	0	—	—	—	3	1	2	—	0	0					
Hiro.2	Hiro.1	33	33	33	0	—	—	32	32	0	—	—	—	18	2	16	—	0	0					
Hiro.3	Hiro.1	14	14	14	0	—	—	13	13	0	—	—	—	12	4	8	—	0	0					
Hiro.4	Hiro.1	22	22	22	0	—	—	22	22	0	—	—	—	11	3	8	—	0	0					
Hiro.5	Hiro.1	41	41	39	2	—	—	37	35	2	—	—	—	25	9	15	—	0	1					
Hiro.6	Hiro.1	51	51	51	0	—	—	51	51	0	—	—	—	26	0	26	—	0	0					
Hiro.7	Hiro.2	33	33	33	0	—	—	27	27	0	—	—	—	14	2	12	—	0	0					
Total		214	214	212	2	—	—	202	200	2	—	—	—	109	21	87	—	0	1					
				(99.1)	(0.9)					(99.0)	(1.0)					(19.3)	(79.8)			(0)	(0.9)			
Hiro.1	Bang.1	18	18	0	6	12	0	12	0	6	—	6	0	3	0	0	—	0	3					
Hiro.2	Bang.1	41	41	0	2	39	0	22	0	2	—	20	0	3	0	1	—	0	2					
Hiro.3	Bang.1	35	35	0	7	28	0	23	0	7	—	16	0	9	0	6	3	0	0					
Hiro.4	Bang.1	2	2	0	2	0	0	2	0	2	—	—	—	2	0	0	—	0	2					
Hiro.5	Bang.1	13	13	0	3	10	0	6	0	3	—	3	0	2	0	1	—	0	1					
Hiro.6	Bang.1	38	38	0	5	33	0	21	0	5	—	16	0	8	0	4	—	0	4					
Hiro.7	Bang.2	60	60	0	0	60	0	31	0	0	—	31	0	15	0	14	1	0	0					
Total		207	207	0	25	182	0	117	0	25	—	92	0	42	0	26	4	0	12					
				(0)	(12.1)	(87.9)	(0)					(0)	(21.4)			(78.6)	(0)			(0)	(28.6)			
Hiro.4	Rano.1	2	2	1	1	—	—	2	1	1	—	—	1	0	0	—	0	1						
Hiro.5	Rano.1	66	52	49	3	—	—	63	46	3	14	—	—	26	1	23	—	0	2					
Total		68	54	50	4	—	—	65	47	4	14	—	—	27	1	23	—	0	3					
				(92.6)	(7.4)					(72.3)	(6.2)	(21.5)					(3.7)	(85.2)			(0)	(11.1)		
Bang.1	Bang.1	62	62	62	0	—	—	57	57	0	—	—	—	34	2	32	—	0	0					
Bang.2	Bang.1	130	30	30	0	—	—	127	27	0	100	—	—	23	3	20	—	0	0					
Total		192	92	92	0	—	—	184	84	0	100	—	—	57	5	52	—	0	0					
				(100)	(0)					(45.7)	(0)	(54.3)					(8.8)	(91.2)			(0)	(0)		
Bang.1	Hiro.1	9	9	—	—	9	0	8	—	—	—	8	0	5	0	5	—	0	0					
Bang.2	Hiro.1	1	1	—	—	1	0	1	—	—	—	1	0	0	—	—	—	0	0					
Bang.2	Hiro.2	10	10	—	—	10	0	9	—	—	—	9	0	4	0	4	—	0	0					
Total		20	20	—	—	20	0	18	—	—	—	18	0	9	0	9	—	0	0					
								(100)	(0)					(100)	(0)			(0)	(100)			(0)	(0)	
Rano.1	Rano.1	136	21	21	0	—	—	133	18	0	115	—	—	49	11	34	4	0	0					
				(100)	(0)					(13.5)	(0)	(86.5)					(22.4)	(69.4)	(8.2)			(0)	(0)	
Rano.1	Hiro.1	23	23	23	0	—	—	23	23	0	—	—	—	19	2	16	1	0	0					
Rano.1	Hiro.2	58	30	30	0	—	—	58	30	0	28	—	—	26	1	24	1	0	0					
Total		81	53	53	0	—	—	81	53	0	28	—	—	45	3	40	2	0	0					
				(100)	(0)					(65.4)	(0)	(34.6)					(6.7)	(88.9)	(4.4)			(0)	(0)	
Rano.1	Bang.1	71	71	—	—	71	0	49	—	—	—	49	0	3	0	2	1	0	0					
								(100)	(0)					(100)	(0)			(0)	(66.7)	(33.3)			(0)	(0)

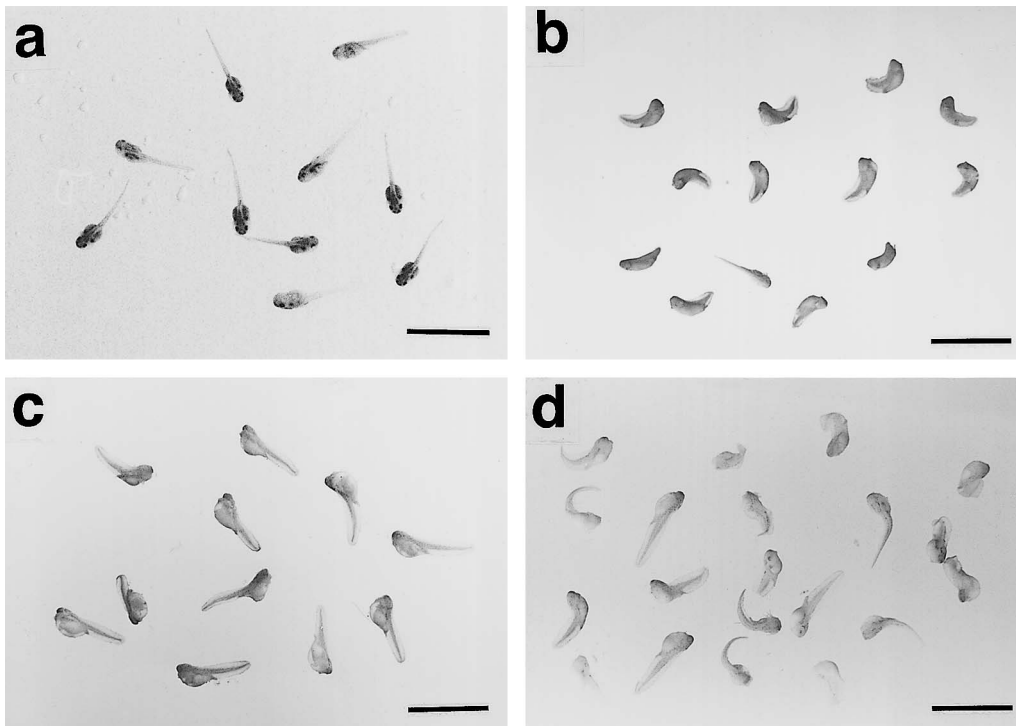


Fig. 7. Hatching-stage tadpoles, including normal offspring from a control cross (a) and abnormal hybrids (b–d) among four populations of *F. limnocharis*. Scale bars=10.0 mm. (a) Rano. ♀ × Rano. ♂, (b) Rano. ♀ × Bang. ♂, (c) Rano. ♀ × Sri ♂, (d) Hiro. ♀ × Sri ♂.

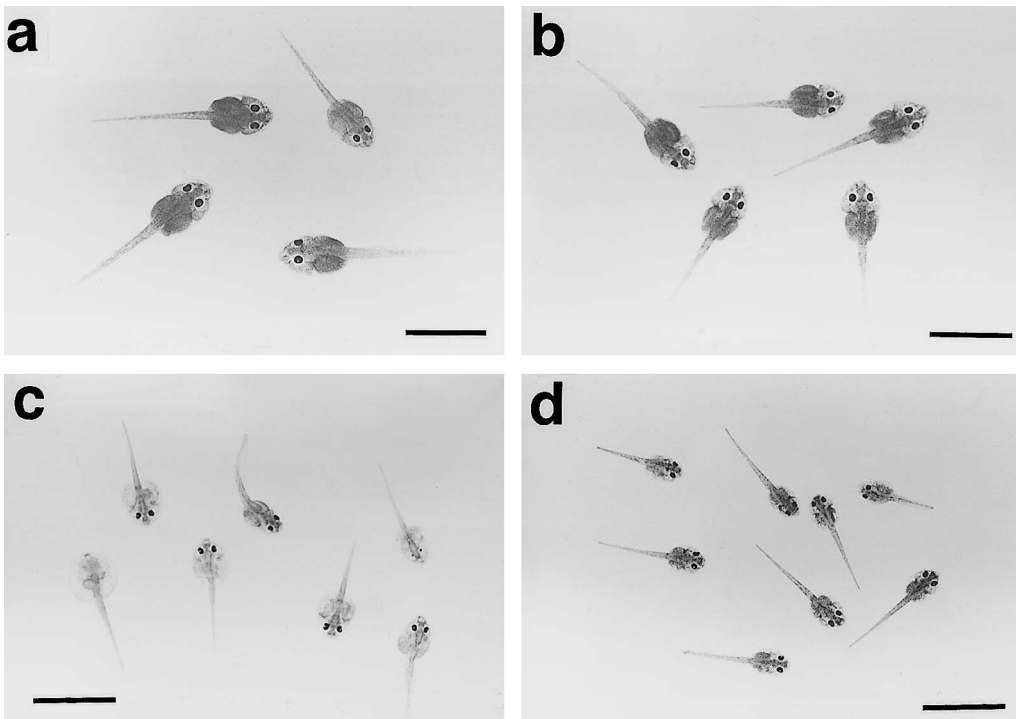


Fig. 8. Feeding-stage tadpoles, including normal offspring from two control crosses (a, b) and abnormal hybrids (c, d) among three populations of *F. limnocharis*. Scale bars=10.0 mm. (a) Hiro. ♀ × Hiro. ♂, (b) Bang. ♀ × Bang. ♂, (c) Hiro. ♀ × Bang. ♂, (d) Bang. ♀ × Sri ♂.

several comparisons including the Ranong and Bangkok populations (Fig. 3d).

Crossing experiments

Developmental capacity

All of the hybrids between a Sri Lanka male and

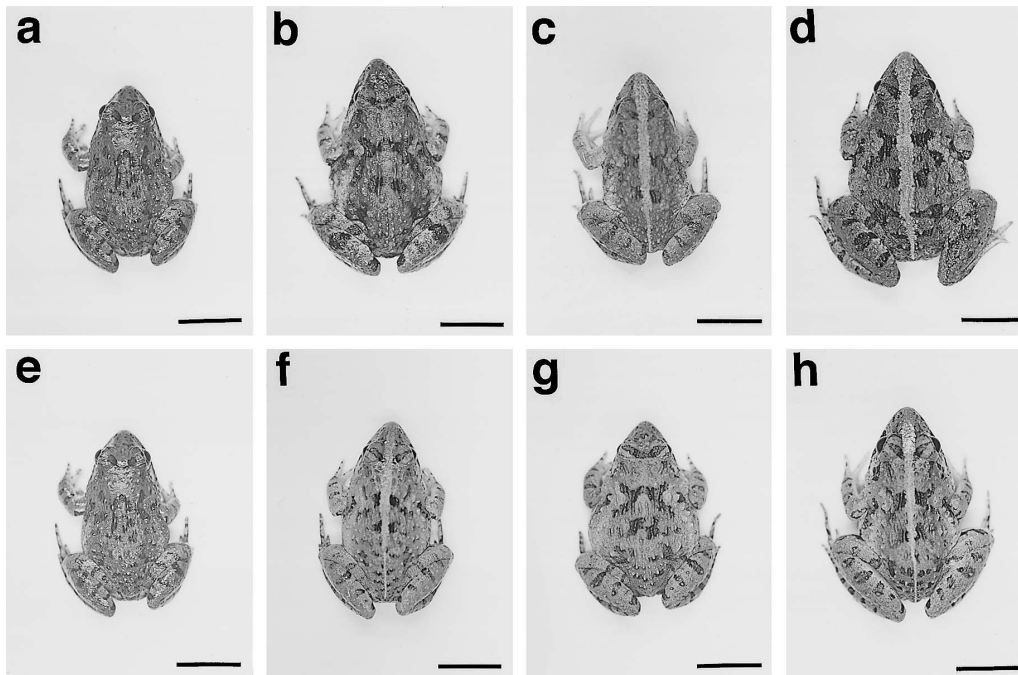


Fig. 9. Dorsal views of 3-month-old offspring from control crosses (a, d, e, h) and from reciprocal crosses between the Hiroshima and Bangkok (b, c) and Hiroshima and Ranong (f, g) populations of *F. limnocharis*. Scale bars=10.0 mm. (a) Hiro. ♀ × Hiro. ♂, (b) Hiro. ♀ × Bang. ♂, (c) Bang. ♀ × Hiro. ♂, (d) Bang. ♀ × Bang. ♂, (e) Hiro. ♀ × Hiro. ♂, (f) Hiro. ♀ × Rano. ♂, (g) Rano. ♀ × Hiro. ♂, (h) Rano. ♀ × Rano. ♂.

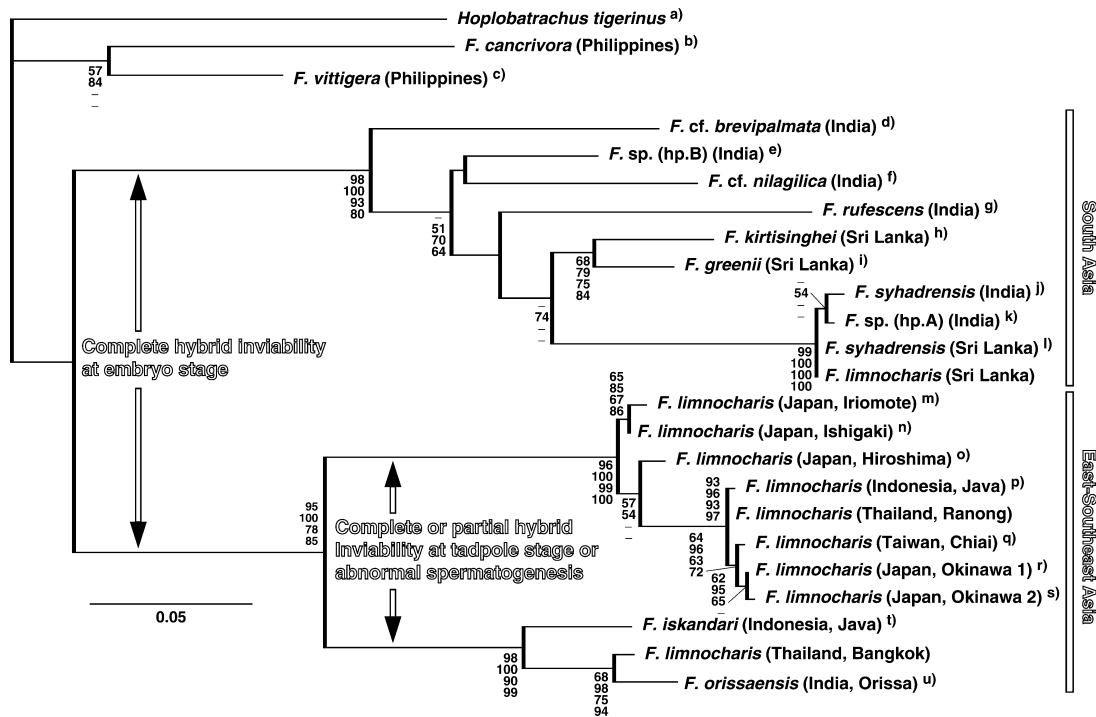


Fig. 10. Phylogenetic tree constructed by the maximum likelihood method, based on nucleotide sequences of a 422-bp segment of the mitochondrial 16S rRNA gene from nine populations of *F. limnocharis*, one population from each of 11 *Fejervarya* species, two populations of *F. syhadrensis*, and one population of *Hoplobatrachus tigerinus* as an outgroup. Numbers near nodes are bootstrap supports (>50%) given in the order of ML, BI, MP, and NJ analyses. The scale bar represents branch length in terms of nucleotide substitutions per site. a) AB167943, Kurabayashi *et al.* (2005); b) AB070738, Sumida *et al.* (2002); c) AY313683, Evans *et al.* (unpublished); d) AB167946, Kurabayashi *et al.* (2005); e) AB167951, Kurabayashi *et al.* (2005); f) AB167949, Kurabayashi *et al.* (2005); g) AB167945, Kurabayashi *et al.* (2005); h) AY014380, Kosuch *et al.* (2001); i) AY014378, Kosuch *et al.* (2001); j) AY841747, Tandon *et al.* (unpublished); k) AB167947, Kurabayashi *et al.* (2005); l) AY141843, Meegaskumbura *et al.* (2002); m) AB070736, Sumida *et al.* (2002); n) AB070735, Sumida *et al.* (2002); o) AB070732, Sumida *et al.* (2002); p) AJ292014, Veith *et al.* (2001); q) AB070737, Sumida *et al.* (2002); r) AB070733, Sumida *et al.* (2002); s) AB070734, Sumida *et al.* (2002); t) AJ292016, Veith *et al.* (2001); u) AY882957, Tandon *et al.* (unpublished).

Hiroshima, Bangkok, and Ranong females died from various abnormalities before the feeding tadpole stage, whereas the controls developed normally (Table 5; Figs. 7, 8). Most of the reciprocal hybrids between the Hiroshima and Bangkok populations and between the Ranong and Bangkok populations died from various abnormalities and underdevelopment during the embryonic and tadpole stages, with only a small number of tadpoles metamorphosing normally, whereas the controls developed normally (Table 5; Figs. 7–9). In contrast, reciprocal hybrids between the Hiroshima and Ranong populations exhibited normal development, like that of the controls (Table 5, Fig. 9).

Ploidy

The control tadpoles for the Hiroshima, Bangkok and Ranong populations were all normal diploids, except for two triploids in the Hiroshima population. Among hybrids between the Hiroshima and Bangkok populations, all the normal tadpoles were triploid, whereas all underdeveloped tadpoles were diploid. Among hybrids between the Hiroshima and Ranong populations, almost all normal tadpoles were diploid, except for four normal triploid tadpoles. Among hybrids between the Bangkok and Ranong populations, all tadpoles were underdeveloped and diploid (Table 6).

Sex

Among the diploid control frogs of the Hiroshima, Bangkok and Ranong populations, 8.8–22.4% were females and the rest were males. It seems that even the control crosses produced an overwhelming majority of males. Among diploid hybrids between the Hiroshima and Bangkok populations, all were males except for several individuals of indeterminate sex, whereas among diploid hybrids between the Hiroshima and Ranong populations, 3.7–6.7% were females and the rest were males. All triploids were males, for both the controls and the hybrids (Table 6).

DISCUSSION

Taxonomic status of the Thailand populations of the *F. limnocharis* complex

Both allozyme and mtDNA sequence analyses revealed that the Bangkok population of the *F. limnocharis* complex from Thailand differed greatly from the Japan and Taiwan populations of this species complex. Between the Bangkok and Hiroshima populations, the 12S and 16S rDNA sequence divergence and Nei's genetic distance were 13.8% and 0.582, respectively. Crossing experiments demonstrated that the Bangkok population is reproductively isolated from the Hiroshima population by partial hybrid inviability at the tadpole stage. Reciprocal hybrids between the Bangkok and Hiroshima populations had abnormal spermatogenesis, even though they were fertile to some extent (Sumida *et al.*, unpublished). Sasa *et al.* (1998) suggested a lower threshold of $D=0.30$ for the evolution of inviability, based on data from 116 crosses involving 46 species of frogs. On the basis of levels of genetic divergence and reproductive isolation, we suggest that both the Bangkok and Hiroshima populations merit specific rank. From our 16S rDNA data, we conclude that *F. limnocharis* from the Bangkok population was allied with *F. iskandari* from Indonesia and *F. orissaensis* from India, but was more closely

related to the latter (Fig. 10).

Fejervarya orissaensis was first described from Orissa State, eastern India, by Dutta (1997). This species is medium-sized (males 36.2–47.2 mm; females 34.2–53.8 mm SVL), with interrupted longitudinal folds on the dorsum, smooth venter, wide inner metatarsal tubercle, and a snout relatively more pointed than that of closely related species, such as *F. limnocharis* from Vietnam, Taiwan, China, and Japan (Dutta, 1997). A description of *F. iskandari* from Cianjur, Java, Indonesia was obtained from Veith *et al.* (2001); it is also medium-sized (five males 40.4–42.7 mm SVL), with a relatively wide head and interorbital space, small tympanum, short forearms, short inner toes, and small inner metatarsal tubercles (Veith *et al.*, 2001). The Bangkok population is also medium-sized (two males 42.0–42.2 mm, and three females 48.0–54.4 mm SVL), with interrupted longitudinal folds on the dorsum and a relatively short hind leg (Fig. 2).

As for the degree of reproductive isolation between the Bangkok population and *F. iskandari* from Indonesia, Djong *et al.* (2007) found that hybrids between them developed normally and reached maturity, but the degree of fertility and the abnormality of spermatogenesis in these hybrids have not yet been examined. On the basis of these data, we conclude that the Bangkok population is closely related to *F. orissaensis* and *F. iskandari*. In the future, the taxonomic status of the Bangkok population should be studied by examining the degree of reproductive isolation and genetic differences from Indonesian *F. iskandari* and Indian *F. orissaensis*.

Hybrids between the Ranong and Hiroshima populations developed normally and reached maturity. Spermatogenesis in mature male hybrids derived from these populations was abnormal to an extent similar to that found in hybrids between the Bangkok and Hiroshima populations, with mostly 2–4 univalents, and rarely 6–12 univalents in chromosomal metaphase spreads (Sumida *et al.*, unpublished). In the allozyme analysis, the Ranong population was closely related to the Bangkok population (Nei's genetic distance $D=0.083$), whereas in the mtDNA analysis it was more closely related to the Hiroshima population (sequence divergence=4.3%). The Ranong population could not be distinguished from the Bangkok population by external morphology or allozyme analysis, but could be clearly distinguished by mtDNA gene sequence analysis (sequence divergence=13.4%). Most of the hybrids between females of the Ranong population and males of the Bangkok population became inviable at the tadpole stage, although a few of them reached metamorphosis and matured. All these hybrids were fertile and almost normal in spermatogenesis (Sumida *et al.*, unpublished). Thus, the Ranong and Bangkok populations may be regarded as separate species from the point of view of mtDNA gene sequence analysis and crossing experiments, but the same species in allozyme analysis and spermatogenesis observations.

These observations lead to questions about the species status of these populations and also the validity of the biological species concept (Mayr, 1969). The biological species concept has been challenged by proponents of several contrasting definitions, including the phylogenetic and evolutionary species concepts, which place more emphasis on phylogenetic history and assessment of the

likely evolutionary “independence” of particular lineages (Hanken, 1999). It should be pointed out that the evolutionary biology of nuclear and cytoplasmic genomes may be different (Powell, 1983). Our allozyme and mtDNA data also imply that the interspecific transfer of mtDNA might possibly have taken place in the Ranong population of Thailand as a result of hybridization similar to that shown in the wild Scandinavian mice studied by Ferris *et al.* (1983). Natural interspecific transfer of mtDNA may have occurred in amphibians via hybrid lineages similar to those reported for the European water frog *Rana esculenta* (Spolsky and Uzzell, 1984), for the fire-bellied toads *Bombina bombina* and *B. variegata* (Vines *et al.*, 2003; Yanchukov *et al.*, 2006), for the *Ensatina* ring-species complex (Moritz *et al.*, 1992; Alexandrino *et al.*, 2005), and for fire salamanders (*Salamandra*) (Garcia-Paris *et al.*, 2003). A more extensive examination is now underway (Kotaki *et al.*, unpublished) to elucidate all aspects of speciation in the *F. limnocharis* complex in Thailand, from the points of view of both the mitochondrial and nuclear genomes, by increasing the sample size and number of populations sampled.

Taxonomic evaluation of the Sri Lanka population of the *F. limnocharis* complex

As for *F. limnocharis* from Sri Lanka, Dutta and Manamendra-Arachchi (1996) described three *Fejervarya* species, *F. limnocharis*, *F. greenii*, and *F. kirtisinghei*. According to them, *F. limnocharis* could be distinguished morphologically from the other two species. *Fejervarya limnocharis* has interrupted longitudinal ridges or rounded tubercles on the skin of the middle of the back, whereas the other two species have uninterrupted longitudinal ridges in the same area. Bossuyt and Milinkovitch (2000) used *F. syhadrensis* from India in their molecular work. Meegaskumbura *et al.* (2002) reported more than 100 species of Old World tree frogs (Rhacophorinae) from Sri Lanka, and revealed upwards of 140 anuran species through an intensive survey, which showed Sri Lanka to be an amphibian hot spot of global importance. They also used *F. syhadrensis* from Sri Lanka for molecular analysis. According to the original description of *F. syhadrensis* by Annandale (1919), who examined specimens from several places in the Satara district, from Khandalla in the Poona district, and from Igatpuri in the Nasik district of India, “the snout–vent length does not exceed 35 mm (27 mm in male and 31.5 mm in female), and the dorsal surface is gray with black spots sometimes with a reddish suffusion; a narrow pale mid-dorsal line often present; the ventral surface is white, with the whole of the throat black in the adult male.” This species is distributed in eastern and western India, Pakistan, and Nepal at low to moderate elevations (Frost, 2004). The snout-vent length of the specimens from Sri Lanka used in our present study was 31.5 mm in the only female and 27.0–29.8 mm in the two males (Table 1), and all displayed rounded tubercles on the dorsal surface (Fig. 2); one male and one female had a broad mid-dorsal line (Fig. 2), but the other male did not. Existing molecular phylogenies suggest that our specimens correspond closely to *F. syhadrensis* from Sri Lanka used by Meegaskumbura *et al.* (2002) and *F. sp.* (hp A) from India described by Kurabayashi *et al.* (2005) (Fig. 10). Tandon *et al.* (unpublished) have also reported that Indian *F. syhad-*

rensis forms a cluster close to our specimens, along with those studied by Meegaskumbura *et al.* (2002). It is reasonable to regard our specimens from Sri Lanka as *F. syhadrensis*. The species named *Fejervarya limnocharis* from Sri Lanka by Dutta and Manamendra-Arachchi (1996) might indeed be possibly referred to *F. syhadrensis*.

Difference in viability of diploid and triploid hybrids

Our study has shown that the Hiroshima and Bangkok populations are reproductively isolated by complete hybrid inviability at the tadpole stage. Almost all hybrid tadpoles from a female of the Hiroshima population and a male of the Bangkok population were underdeveloped and inviable, though a small number of them developed normally and metamorphosed. All of the normally developed tadpoles were found to be triploid. Several triploids were also found among hybrids between the Hiroshima and Ranong populations, and even in the control Hiroshima population, along with many normal diploids. Generally speaking, triploid tadpoles are more viable than diploid ones of the same species (Kawamura, 1952; Nishioka, 1971, 1983). Bogart (1972) also reported that triploid hybrids between distantly related species of *Bufo* were more viable than diploids. Diploid hybrids between female *Rana catesbeiana* and male *R. clamitans* stopped development at the embryonic stage (Elinson, 1977), but 36 allotriploids constructed from two genomes of *R. catesbeiana* and one genome of *R. clamitans* developed normally through to metamorphosis (Elinson and Briedis, 1981). Kawamura and Nishioka (1975, 1977) reported that reciprocal hybrids between *R. nigromaculata* and *R. lessonae* died at the embryonic stage, whereas the allotriploids consisting of two genomes of *R. nigromaculata* and one genome of *R. lessonae* were viable and become mature. Two Japanese brown frog species, *Rana japonica* and *R. tsushimensis*, are isolated by complete hybrid inviability; that is, all diploid hybrids between the two species die at the tadpole stage (Kawamura and Nishioka, 1977; Kawamura *et al.*, 1981). Sumida and Nishioka (1993) found that almost all of the artificially produced allotriploids between *R. japonica* and *R. tsushimensis* developed normally and reached maturity, and that some of them reproduced by “hybridogenesis,” in which the *R. japonica* genome was eliminated during spermatogenesis. In our crosses between individuals of the Hiroshima and Bangkok populations, all diploid tadpoles were underdeveloped and died before metamorphosis, unlike triploid tadpoles. These results also confirm that these two populations are differentiated from each other at the species level, as stated earlier. It is clear that triploid interspecific hybrids produced spontaneously or artificially by suppression of the extrusion of the second polar bodies, and consisting of two genomes from the female parent and one genome from the male parent, are viable through metamorphosis, even though diploid hybrids of the same combination are inviable at the tadpole stage.

Phylogenetic relationships among the *Fejervarya limnocharis* complex in Asia inferred using 16S rDNA data

The ML tree (Fig. 10) shows that members of the *Fejervarya* species complex, excluding *F. cancrivora* and *F. vittigera* from the Philippines, generally diverged into two groups, the South Asian and East-Southeast Asian groups

(BP=80–100% and 78–100%, respectively), although *F. orissaensis* from India was included in the East-Southeast Asian group. Basal affinities and thus monophyly of the species complex were unclear, as bootstrap values were low. The East-Southeast Asian group was divided into two subgroups, one including three *F. limnocharis* clades: (1) Iriomote and Ishigaki (Japan), (2) Hiroshima (Japan), and (3) Java (Indonesia), Ranong (Thailand), Chiaï (Taiwan), and Okinawa (Japan), and the other including *F. iskandari* from Java (Indonesia), *F. limnocharis* from Bangkok (Thailand), and *F. orissaensis* from Orissa (India). These were designated as the *F. limnocharis* and *F. iskandari* subgroups, respectively.

The South Asian group formed a cluster distantly related to the East-Southeast Asian group referred to above, and then diverged into a number of species whose identification is not valid, including *F. cf. brevipalmata*, *F. sp.* (hp. B of Kurabayashi *et al.*, 2005), *F. cf. nilagilica* and *F. rufescens* from India, *F. kirtisinghei* and *F. greenii* from Sri Lanka, and *F. syhadrensis* from India and Sri Lanka (Fig. 10). Our crossing experiments suggest that these two groups are reproductively isolated from each other by complete hybrid inviability at the embryonic stage. However, within the East-Southeast Asian group, the *F. iskandari* and *F. limnocharis* subgroups seem to be isolated from each other by partial hybrid inviability at the tadpole stage, and also by abnormal spermatogenesis (Fig. 10).

Within the South Asian group, several species seem to be isolated from one another by complete hybrid sterility (Islam *et al.*, unpublished). The extent of sequence divergence between the South and East-Southeast Asian groups was 19.3–21.9% ($x=21.2\%$). Application of a previously published estimate of the evolutionary rate (0.69% change per million years) for amphibian mitochondrial genes (Macey *et al.*, 1998; Shaffer and McKnight, 1996) suggests that the South and East-Southeast Asian groups diverged 14–16 mya. Genetic distances estimated from allelic frequencies, 1.185–1.898 ($x=1.506$), demonstrated extreme divergence between the South and East-Southeast Asian groups. Applying an amphibian-specific empirical calibration of an allozyme clock (Maxson and Maxson, 1979) of 1.0 Nei's (1972) $D=14$ my, the present allozyme data suggest that these two groups diverged 8.3–13.3 mya. Tandon *et al.* (unpublished) reported that *Fejervarya limnocharis* of East Asia diverged approximately 12 mya from *F. syhadrensis* of South Asia, on the basis of mtDNA sequence divergences.

The divergence times deduced from mtDNA and allozyme data roughly correspond to geological events that effectively separated the Indian subcontinent from the rest of Asia. The distribution of the *F. limnocharis* complex mostly in East Asia indicates that this complex separated from the South Asian populations after the formation of Himalayas and the uplift of Tibetan Plateau in the middle Miocene, between 15 and 5 mya. Thus the diversification of this complex apparently occurred entirely within the Miocene, before the Himalayan foothills arose in the Pliocene. The date of 15 mya corresponds to increased aridity in South-East Asia caused by the uplifting of the Transhimalaya and the Tibetan Plateau (Harrison *et al.*, 1992). The uplift of Himalayas and the extensive embayment in Assam and Myanmar provided a barrier to the *Fejervarya limnocharis* complex between

India and the rest of Eurasia in the later Tertiary. Further extensive studies using molecular analyses and crossing experiments will be needed to elucidate all aspects of the speciation and phylogeny of the genus *Fejervarya*, involving specimens from throughout its area of distribution in Asia.

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